Managing Hydrilla: Field Trials Using Biological and Chemical Control Technologies Alone and in Combination

PURPOSE: This technical note describes the results of two field studies to evaluate the effectiveness of a granular formulation of the fungal pathogen *Mycoleptidiscus terrestris* (Gerd.) Ostaseski (*Mt*), against the noxious aquatic weed, hydrilla (*Hydrilla verticillata* (L.f.) Royle). The efficacy of integrating this biological control agent with herbicides to improve long-term management of hydrilla was also investigated.

Study 1: *Mt* Formulation Field Trial

BACKGROUND: The endemic fungal pathogen *Mt* is a potential biological control agent for hydrilla as demonstrated by laboratory, greenhouse, and field trials (Joye 1990, Joye and Cofrancesco 1991, Joye and Paul 1992, Shearer 1996, 1997). It has also been demonstrated to have excellent possibilities for management of hydrilla when used in combination with low rates of the herbicide fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl) phenyl]-4 (1H)-pyridinone) (Netherland and Shearer 1996; Nelson, Shearer, and Netherland 1998). To further develop the fungus for use in the field and as a potentially marketable product, it must undergo formulation.

The formulation process involves a number of steps including fermentation, design of biocarriers, testing fungal and biocarrier compatibility, incorporation of the fungus into the biocarrier, extrusion, milling, and drying. The time required to develop a final product may vary because each step in the formulation process must be refined and changes in one step may in turn affect other steps. To date, little research has been directed toward the development of formulated microbial organisms for use in aquatic systems. To be effective in an aquatic environment, the shape, size, buoyancy, and adhesion properties of a formulation may need to be adjusted to allow for dispersal and adequate coverage of the target plant in an aqueous medium. Trans America Product Technology, Inc. (TAPT, St. Charles, MO) was contracted to develop a prototype granular formulation of *Mt* by incorporating the fungus into a patented EPA-approved biocarrier, Biocar 405.

In small-scale greenhouse trials, the prototype formulation proved efficacious and significantly reduced hydrilla shoot biomass at 4 weeks post application (Shearer 1998). During 1998, the quantity of granular product necessary to adequately conduct field trials required major alterations to the formulation process from that used in developing the prototype. For example, the fermentation was upgraded from 1-L shaker flasks to 20-L industrial fermentors, the fermentation medium was changed from Richard's V-8 broth to a Biocar-based broth, the extruder was upgraded from a small hand-cranked unit to a large automatic unit, and the new formulation was dried in approximately 6 hr versus 24 to 48 hr. Time constraints did not allow laboratory evaluation of the new formulation prior to field testing to determine if changes in the process affected performance of the formulation. This study reports the results of field trials using *Mt* formulation and *Mt* slurry on hydrilla planted in ponds at the Lewisville Aquatic Ecosystem Research Facility (LAERF) in Lewisville, TX.

MATERIALS AND METHODS: Seed cultures were prepared by inoculating 1-L Erlenmeyer flasks containing Richard's V-8 juice broth (glucose, 10 g; KNO_3, 10 g; $CaCO_3$, 3 g; V-8 juice (Campbells), 200 ml; H_2O, 800 ml) with *Mt* plugs cut from the leading edge of actively growing colonies. The flasks were agitated on a gyrotory shaker set at 200 rpms. After 6 days, the mycelial pellets were ground to a slurry, decanted into sterile 1-L plastic bottles, and shipped overnight to TAPT. The slurry was used to inoculate 20-L fermentors containing Biocar 405, nutrients (dextrose, 20 g/L; KNO_3, 20g/L), and sterile water as the broth medium. After 4 days of fermentation, the mycelial pellets were harvested and incorporated into Biocar 405 plus sterile water. The formulation was then extruded, dried, and milled into a 20- to 40-mesh granule. Immediately following milling, the formulation was refrigerated and kept cool until application at the field site.

Mt slurry for the field test was prepared as described above for the seed cultures. Following agitation on the gyrotory shaker for 6 days, the flask contents were filtered through four layers of cheesecloth, reconstituted with sterile water, and comminuted in a blender for 30 sec. The slurry was refrigerated until application at the field site.

Subsamples of *Mt* slurry and formulation were analyzed for viability and pathogenicity. Viability was determined by serial dilution. The dilutions were plated onto Martin's agar and *Mt* colonies (cfus) were counted after 5 days. Hydrilla plants grown in 12-L columns were treated with 5 ml and 10 ml of *Mt* slurry and 5 g and 10 g of formulated *Mt*. Untreated plants were used as controls. Treatments were replicated three times. Plants were observed over a 21-day period and assigned disease ratings based on a 0 to 4 scale: 0 = no macroscopic damage; 1 = slight chlorosis of leaves; 2 = general chlorosis of leaves and stems; 3 = general chlorosis and wilting of leaves and stems; 4 = total collapse or disruption of plant tissue. Aboveground biomass was collected from untreated controls and from slurry- and formulation-treated plants that showed disease symptoms and was dried at 60 °C to a constant weight.

Twenty-one treatment plots were set up in a hydrilla-planted pond prior to treatment. Plots (4 m by 4 m by 1 m) were enclosed with plastic and secured at the pond bottom to prevent cross-contamination. The partitions allowed for increased replication and assessment of *Mt* slurry and formulation rates (Table 1). The inoculum was applied by distributing it over the water surface within the plot and allowing it to naturally dissipate onto plant surfaces.

Table 1
Proposed Treatment Rates for Application of Mt Formulation to 4-m by 4-m by 1-m Plots of Hydrilla[1]

Application Rate, kg/ha	Kilograms per Plot Required	Kilograms for Triplicate Tests
560	2.21	6.64
280	0.89	2.66
112	0.44	1.33
56	0.22	0.66
22	0.09	0.27

[1] The above calculations are based on a formulated product rated at 1×10^5 cfus/g

Plant and water samples were collected from the plots at 0, 1, 7, and 21 days after treatment (DAT) to determine the level of *Mt* infection of the plant tissue and dissipation of fungal residues in the water column. The samples were kept cool and transported to the biomanagement laboratory at the Waterways Experiment Station, Vicksburg, MS, for processing.

Pretreatment and postreatment shoot biomass was collected from the pond and dried at 60 °C to a constant weight. Samples were collected by harvesting the shoot biomass contained within a 0.25-m^2 quadrat. A single sample was collected outside the perimeter of each treatment plot for pretreatment biomass. Posttreatment samples were collected at three random locations within a treatment plot. Samples were not collected from formulation-treated plots if hydrilla was not visually impacted by the treatment.

RESULTS AND DISCUSSION: Application rates of formulation and slurry required for the field test were based on an *Mt* viability count of 1×10^5 cfus/g (Table 1) and 1×10^6 cfus/ml, respectively. At the time of application, the granules had a viability count of 1×10^4 cfus/g, a tenfold reduction from that measured prior to shipment to the LAERF. Exposure to extreme heat during shipment in all probability contributed to the reduced viability. The fungal slurry was kept cool and maintained an acceptable viability count of 1×10^6 cfus/ml at the time of application.

Disease symptoms were evident on hydrilla treated with the fungal slurry within 5 DAT both in the columns and in the pond. Plants treated with formulation granules remained asymptomatic even at the highest dosage rate. This was unlike previous tests in which formulation granules induced chlorosis and flaccidness at points of contact with the plant (Shearer 1998). At 21 DAT, slurry-treated plants had mean disease ratings of 3.5 and 4, respectively, for the 5-ml and 10-ml applications whereas formulation-treated plants were asymptomatic. Shoot biomass of hydrilla in the 12-L columns was reduced 83 percent and 91 percent by the 5-ml and 10-ml slurry treatments, respectively, compared to untreated controls (Table 2). From results of previous studies, it was expected that the 5-g and 10-g formulation treatments would reduce shoot biomass by a similar amount (Shearer 1998).

Table 2
Mean Dry Weight of Hydrilla Shoot Biomass Collected 21 DAT with a Fungal Slurry of Mt in Pond and Column Studies

Treatment	Pond Hydrilla biomass/g	Column Hydrilla biomass/g
Control	61.70	8.33
Fungal slurry (5 ml) (416 cfus/ml)	--	1.48
Fungal slurry (10 ml) (833 cfus/ml)	--	0.76
Fungal slurry (11 liters) (687 cfus/ml)	6.42	--

There was no visible evidence of disease development on hydrilla in the formulation-treated plots at 21 DAT; therefore, biomass samples were not harvested. The slurry reduced hydrilla shoot biomass by 89 percent compared to untreated controls (Table 2). Percent biomass reductions in the pond and greenhouse column studies were similar (Table 2).

Extreme heat in the LAERF area during the study was a concern because high temperatures can adversely affect viability of microorganisms. On agar media, *Mt* growth is inhibited around 33 °C.[1] The temperatures in the pond were between 30 and 35 °C at the water surface and moderated slightly with depth. These temperatures did not appear to adversely affect the fungal slurry because it was able to incite disease resulting in a reduction in shoot biomass. The field results were verified in greenhouse tests where water temperatures in the columns did not exceed 30 °C. Therefore, temperature was probably not a factor in the lack of disease development in formulation-treated plots.

Mt was not present in plant or water samples collected prior to treatment. One day after treatment, *Mt* was detected in the water of almost all the treatment plots. *Mt* was also found in one control plot, indicating a minimal amount of cross contamination. At 7 and 21 DAT, the fungus was no longer detectable in the water. At 1 DAT, *Mt* was found in plant samples in two of the three plots treated with fungal slurry and in one plot treated with formulation at a rate of 280 kg/ha. *Mt* was not found in plant samples collected at 7 and 21 DAT.

The lack of disease development in the hydrilla treated with the formulation was unexpected. Although application rates were below levels that had produced excellent results in previous greenhouse tests and the granules had reduced viability, diseased areas should have been evident at contact points between the granules and the plant. When production of the formulation began in early summer, several significant changes were made in procedures and equipment that differed from those used in formulating the prototype. All of these changes were initiated over a short time period without evaluation of their potential effects on the performance of the formulation. The changes did not seem to affect fungal viability but it is not known how the changes affected other aspects of fungal growth and metabolism. For example, relatively simple changes in fermentation medium (differences in micro nutrients, sugar levels, or carbon/nitrogen ratios) can alter fungal growth, sporulation, production of secondary metabolites, or virulence.

Why the formulation failed to incite disease in hydrilla in the field tests at the LAERF is at present unknown. The fungal component delivered as a slurry seemed to perform in a manner consistent with previous field tests. In order to pinpoint the exact problem, it is recommended that all facets of the formulation process be reevaluated. Initial testing can be done in the laboratory by repeating procedures used in the prototype formulation. Once an efficacious granular formulation has been produced, each facet of the formulation process should be examined, including fungal preparation, fermentation methods and media, biocarriers, extrusion equipment and techniques of drying, milling, and storing.

Study 2: Integrating *Mt* with Herbicide Field Trial

BACKGROUND: Various practices reduce aquatic weed populations: mechanical harvesting; release of biological agents (insects, herbivorous fish, fungal pathogens); application of chemical herbicides; and water manipulation (drawdowns). In addition to these traditional management methods, recent research has shown that integrating a sublethal herbicide rate with a plant-specific pathogen offers a new and innovative control strategy in the fight against nuisance aquatic weeds.

[1] Unpublished data, 1997, Dr. Judy Shearer, Research Pathologist, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

Nelson, Shearer, and Netherland (1998) showed that application of the endemic fungal pathogen *Mt* (100 to 200 cfus/ml) along with 5 µg/L fluridone controlled 93 percent of the hydrilla biomass growing in outdoor tanks, whereas either product used alone reduced hydrilla growth by only 40 percent. In addition, using fluridone at such low rates had minimal impact on nontarget plant species such as vallisneria (*Vallisneria americana* Michx.) and American pondweed (*Potamogeton nodosus* Poiret).

As a bioherbicide, *Mt* is similar to a contact chemical herbicide, causing localized damage to plant tissue where contact occurs. With any type of contact agent (biological or chemical), translocation of the product inside the plant does not occur; therefore, regrowth from surviving root stock can occur. Once formulated as a marketable product, *Mt* would act similar to a contact herbicide, with the added advantage of hydrilla specificity. Where nonselective, contact chemical products are prohibited or undesirable due to potential non-target effects, *Mt* would offer aquatic plant managers another option for selective nuisance plant control. Furthermore, combining the pathogen with low rates of the systemic herbicide fluridone produces a beneficial synergistic effect that enhances the use of both of these products.

The objectives of this study were to evaluate the efficacy of integrating *Mt* as a granular formulation with the herbicide fluridone and to compare this combined treatment with an integrated treatment of fluridone with a contact herbicide, copper, against the target weed hydrilla.

MATERIALS AND METHODS: This study was conducted in a pond at the LAERF that had been planted with hydrilla. Enclosed test plots (4 m by 4 m) were established in thick hydrilla stands using plastic barriers that were secured to each plot corner marker and to the pond bottom. The plastic extended above the water surface to prevent cross-contamination of treatments between plots. For this study, untreated control plots were established in a similar hydrilla stand growing in an adjacent pond to eliminate any possible risk of chemical and fungal pathogen contamination.

The target treatment rates included 112 kg/ha *Mt* + 5 µg/L fluridone, 280 kg/ha *Mt* + 5 µg/L fluridone, 5 µg/L fluridone + 0.1 mg/L copper, 5 µg/L fluridone + 0.25 mg/L copper, and untreated controls.

The granular *Mt* formulation developed and used in Study 1 was also used in this field trial. The formulation was applied by hand-tossing the granules evenly onto the water surface. Integrated treatments were applied by dispensing both agents simultaneously to each designated plot. Copper was applied as the liquid commercial formulation Nautique^{R} (110.0 g elemental copper (active ingredient) per liter). The target rate of fluridone (5 µg/L) was achieved by applying a combination of two fluridone formulations: Sonar^{R} AS and Sonar^{R} SRP. Sonar^{R} AS is a liquid formulation (479 g active ingredient per liter) that was used to bring the initial water concentration up to the designated target rate. Sonar^{R} SRP, a slow-release pellet formulation, was applied at a total concentration of 20 µg/L with the intent of maintaining fluridone water concentrations at the target 5 µg/L rate. To monitor fluridone concentrations in the water, 1-L water samples were collected at 1, 4, 7, 14, 28, and 42 DAT and analyzed for residues. At each sampling interval, three water samples were collected mid-depth in the water column at random locations within fluridone-treated plots. Residue analyses were conducted using a high-performance liquid chromatography (HPLC) technique (Lily Research Lab 1980). Hydrilla biomass was sampled 21 and 42 DAT using a 0.25-m^2 quadrat as

described in Study 1. Biomass was dried to a constant weight at 60 °C and dry weights were recorded.

Treatments were randomly assigned to plots and were replicated three times. Data were subjected to analysis of variance (ANOVA) procedures and treatment means were compared using Fisher's protected Least Significant Difference (LSD) test at the 0.05 level of significance.

RESULTS AND DISCUSSION: Residue analysis showed that the initial target fluridone concentration of 5 µg/L was achieved (Table 3). Aqueous fluridone concentrations fluctuated slightly during the study; however, when averaged over the 42-day test period, fluridone concentrations were maintained near 6 µg/L. Variations in fluridone water concentrations over time are due to the release properties of the SRP formulation.

Table 3
Fluridone Residues in Water at Three Random Locations Following Treatment with 5-µg/L Fluridone as the Sonar^{R} AS Formulation plus 20-µg/L Fluridone as the Sonar^{R} SRP Formulation

Days after Treatment	Fluridone Concentration (µg/L			
	Sample 1	Sample 2	Sample 3	Mean
1	4.4	5.3	6.1	5.3
4	5.7	6.1	6.4	6.1
7	8.1	7.4	7.3	7.6
14	12.0	10.4	11.2	11.2
28	5.3	5.1	4.8	5.1
42	2.4	2.1	1.9	2.1

Treatment effects on hydrilla biomass are reported in Figure 1. Twenty-one days after treatment, only the fluridone + copper treatments showed significantly lower biomass compared to untreated plants. Biomass reductions averaged 88 percent. Statistically there was no difference between the two fluridone + copper treatments. Similar to Study 1, the granular formulation of *Mt* was not effective on hydrilla. There were no visible symptoms of fungal infection on hydrilla stem and leaf tissues. Previous studies have reported disease symptoms within 5 days following application with *Mt* as a mycelial slurry (Joye and Cofrancesco 1991, Shearer 1996).

By the final biomass sampling 42 DAT, all treatments showed significantly lower biomass compared to untreated plants. Fluridone + 0.25 mg/L copper showed the most dramatic effect; decreasing plant mass by 98 percent compared to untreated hydrilla. Statistically, there were no differences between the two rates of copper when combined with fluridone. The fluridone + *Mt* treatments reduced plant biomass by an average of 59 percent compared with untreated controls. Since *Mt* could not be reisolated from plants treated with the granular formulation in Study 1, the effects observed with fluridone + *Mt* in this study were presumed to be solely from fluridone. A similar reduction in hydrilla biomass was observed by Nelson, Shearer, and Netherland (1998) when plants were treated with a static exposure of 5-µg/L fluridone alone.

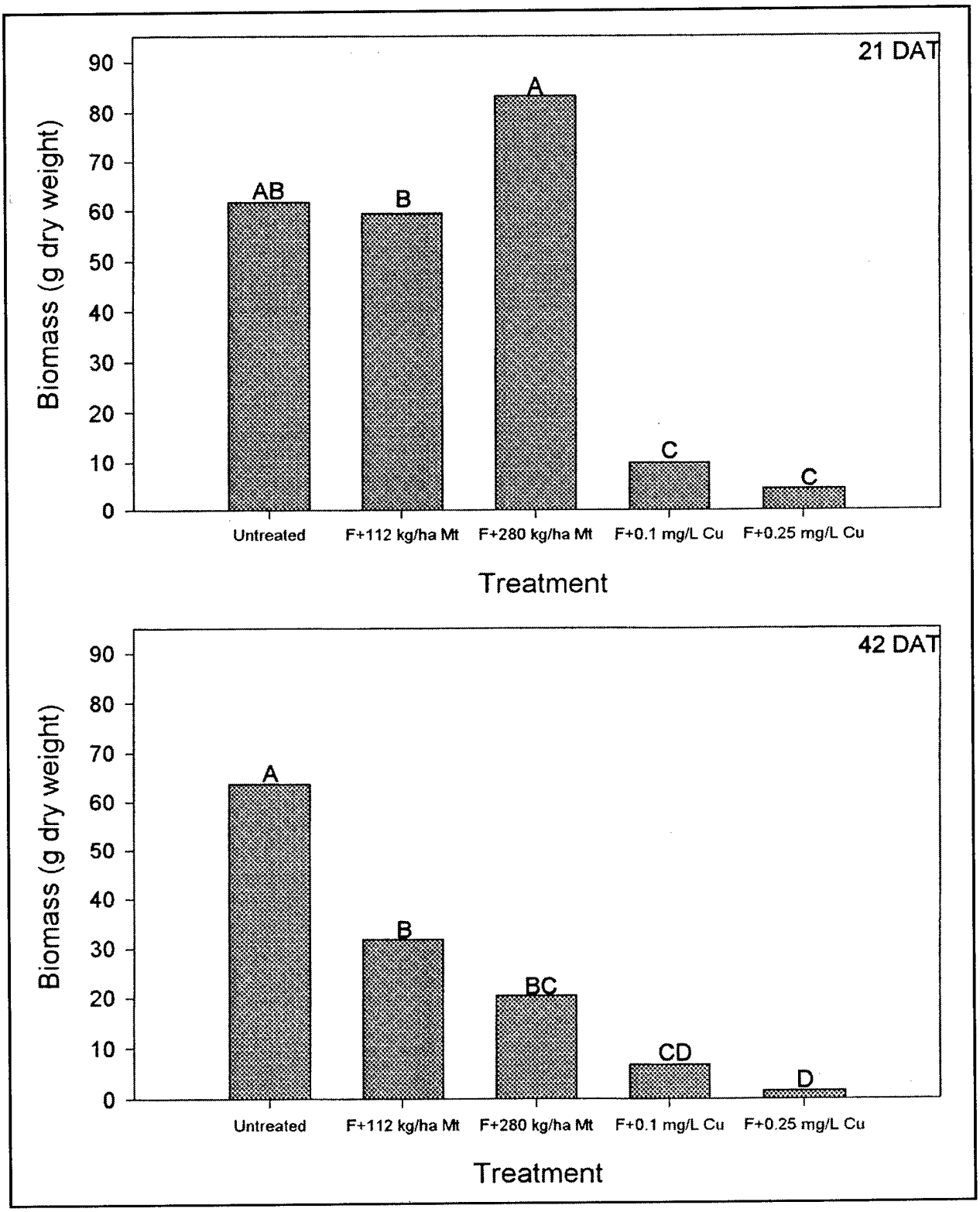

Figure 1. Mean dry weight biomass of hydrilla at 21 and 42 days after treatment following application of integrated treatments of *Mt* + fluridone (F = 5 µg/L) and fluridone + copper. Letters above bars denote statistical differences at $P \leq 0.05$ according to Fisher's protected LSD test

Since the granular *Mt* was ineffective, the effects of a contact bioherbicide could not be compared with those observed with the contact chemical herbicide. However, the data did show that integrating low rates of two chemical products - a contact herbicide and a systemic herbicide - provided better hydrilla control than the sustained low rate of the systemic herbicide fluridone acting alone (as observed in the *Mt* + fluridone treatment). Integrating control practices allows aquatic plant managers to better utilize the available tools for controlling nuisance plant infestations, and thus improves overall management efficiency.

FUTURE WORK: Future research will focus on evaluating other potential herbicide-pathogen combinations against hydrilla and another nuisance submersed plant species, Eurasian watermilfoil (*Myriophyllum spicatum* L.). In addition, the development of a suitable granular formulation of *Mt* will continue.

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